Original Article
Particulate matter increases connexin 43 expression and exacerbates endothelial barrier disruption

Jun Zhang[1,2*], Xiaomin Wu[1*], Ying Liang[1*], Gabriel Kelly[1], Janis M Burt[3], Liming Zhang[2], Ting Wang[1,4]

[1]Department of Medicine, University of Arizona, Tuscon, AZ, USA; [2]Department of Respiratory and Critical Care Medicine, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China; [3]Department of Physiology, University of Arizona, Tuscon, AZ, USA; [4]Center of Translational Science, Florida International University, 11350 SW Village Parkway, Port St. Lucie, FL, USA. *Equal contributors.

Abstract: Objectives: Particulate Matter (PM) air pollution is known to exacerbate cardiopulmonary diseases. We previously demonstrated that PM mediates endothelial injury and barrier disruption by modulating the endothelial cytoskeleton and cell-cell junctions, but the effects of PM exposure on cell-cell communication and gap junction activity are still unknown. Methods: This study focused on the characterization of PM-regulated endothelial dysfunction through connexin 43 (Cx43), the most abundant gap junction protein expressed in lung endothelial cells (ECs), using cultured human lung endothelial cells and a well-characterized PM sample. Results: PM exposure induced a time-dependent increase of Cx43 in human lung ECs at both the mRNA and protein levels. N-acetylcysteine (NAC), a reactive oxygen species (ROS) scavenger, significantly suppressed PM-induced Cx43 expression. Cx43 proteins on the plasma membrane and ER/Golgi apparatus were elevated in response to a PM challenge. In addition, PM induced gap junction activity, which was indicated by green fluorescence dye transfer between two adjacent ECs. Moreover, GAP27, a selective Cx43 channel inhibitor, attenuated PM-induced human lung EC barrier disruption, which was reflected by rescued trans-endothelial electrical resistance (TER) with an electric cell-substrate impedance sensing system. Moreover, knocking down Cx43 alleviated PM-induced myosin light chain (MLC) phosphorylation. Conclusions: These results strongly suggest that Cx43 plays a key role in PM-mediated endothelial barrier disruption and signal transduction. Cx43 may be a therapeutic target in PM-mediated cardiopulmonary disorders.

Keywords: Particulate matter, gap junction, connexin 43, endothelial cell, barrier function

Introduction

Airborne particulate matter (PM) is a complex mixture of solid and liquid particles suspended in gas [1]. Epidemiologic studies showed that PM are associated with increases in morbidity and mortality, while also contributing to respiratory symptoms and heart disease [2]. In addition to leading to cardiovascular risk [3], inhalation of airborne PM generates a wide range of respiratory health effects, such as asthma, lung function deterioration, lung cancer, and chronic obstructive pulmonary disease (COPD) [4].

PM triggers multiple cellular responses in the respiratory system, including innate immunity, adaptive immunity, and the production of reactive oxygen species (ROS), which lead to pulmonary anatomic and physiologic remodeling [5]. Our lab has performed several studies to elucidate the mechanisms of PM-mediated vascular barrier dysfunction. Exposure of a murine asthma model to PM produced significant changes in airway hyperresponsiveness and stimulated a strong molecular signature of inflammatory/asthmatic gene expression [6]. Furthermore, using a monolayer human lung endothelium system, we verified that PM directly disrupted pulmonary endothelial cell (EC) barrier integrity through ROS-dependent P38 Mitogen-activated protein kinases (MAPK) activation [7, 8] and calpain-mediated tight junction protein degradation [9], thereby contributing to acute inflammatory lung hyperpermeability in vivo.

Gap junctions are membrane channels that regulate intercellular communication by permitting the exchange of ions (electrical communication) and small cell signaling molecules (chemical communication) [10]. These channels are composed of two hemichannels, in a homo or hetero manner, termed a connexon that oligomerizes with six connexin proteins [11]. It is widely accepted that alterations in gap junction organization and connexin expression are critical to vascular [12] and pulmonary inflammatory diseases [13].

Connexin 37 (Cx37), Connexin 40 (Cx40), and Connexin 43 (Cx43) are the predominant connexins expressed by pulmonary endothelial cells in mammals [14], and altered expression of these proteins contributes to the pathogenesis of acute lung injury, asthma, cystic fibrosis, idiopathic pulmonary fibrosis, and pulmonary hypertension [13, 15-17]. Exposure to multiple inflammatory stimuli, thrombin, Lipopolysaccharide (LPS) and cyclic stretch upregulates Cx43 expression in human pulmonary endothelial cells, resulting in changes in EC barrier function [18]. PM is a well-known risk factor that increases EC permeability; however, it is not known whether gap junction proteins participate in PM-induced cell signaling.

In the present study, we found that Cx43 was upregulated by PM exposure at both the mRNA and protein levels, and the truncated isoform at 20 kDa assisted Cx43 protein assembly between apposing cell plaques for gap junction channel formation through the Endoplasmic Reticulum (ER)/Golgi vesicular transport pathway. The increase in Cx43 expression was blocked by an ROS scavenger. Functional analyses of intercellular communication indicated that PM treatment increased Cx43 channel activity. In addition, inhibiting Cx43 activation (siRNA knockdown) alleviated PM-induced myosin light chain (MLC) phosphorylation (specific inhibitor peptide or siRNA knockdown) and attenuated PM-induced EC barrier dysfunction. Our results indicated that Cx43 mediated PM-induced EC barrier dysfunction through ROS-dependent regulation.

Material and methods

PM

PM samples (0.1-0.3 μm of aerodynamic diameter) were collected (April 2005) from Ft. McHenry Tunnel, Baltimore, MD, using a high-volume cyclone collector [7, 19]. No detectable endotoxin (LPS) contamination was found in the PM suspension (1 mg/ml) in water by an LPS Enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science). The elemental composition (micrograms per gram) has been described previously [9].

Human endothelial cell culture

Human lung ECs were purchased from Lonza and cultured in endothelial growth media 2 (EGM2) complete medium (Lonza) according to the protocol as described previously [7]. All experiments were conducted using cells at passages 6-8. ECs were grown to 70% confluence and then transfected with Cx43-targeted siRNA (Dharmacon) by using siPORT Amine reagent (Ambion). After incubation with siRNA for 4 hr, the medium was changed to fresh complete culture medium for transfected cells, and the cells were cultured for 24 hr before use in subsequent experiments.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technology) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 mg of DNase I (Thermo)-treated RNA using the random primers p(N)6 and Maxima H Minus Reverse Transcriptase (Thermo). One microliter of the first-strand cDNA reaction was used as a template for real-time PCR using Taqman Probes and Universal Master Mix II (Life Technology). The GAPDH gene was used as an internal control. Real-time PCR was performed by using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All reactions were performed in triplicate.

Western blot analysis

ECs were lysed with RIPA buffer and quantified using a BCA Protein Assay (Pierce). Twenty micrograms of protein were separated on 4-12% Bis-Tris Plus gels (Life Technology) and transferred to a nitrocellulose membrane (Bio-Rad). After being blocked with 5% BSA, the membrane was incubated with Cx43 C-terminal-specific antibodies (Abcam) or GAPDH antibodies (Pierce) prepared in 5% BSA buffer. Signals were visualized using a chemiluminescence detection kit (Thermo Fisher Scientific) and myECL imager (Thermo Fisher Scientific). The

intensity of the individual bands was quantified using ImageJ software.

Transendothelial electrical resistance

The transendothelial electrical resistance (TER) assay was conducted as previously described [20]. Endothelial cells were seeded on gold microelectrodes and grown to confluence. TER was measured using an electrical cell-substrate impedance sending system (ECIS) (Applied Biophysics). The TER tracings represent resistance records, and reduced resistance indicates an increase in EC barrier disruption.

Immunofluorescence analysis

Human lung ECs were plated on collagen-coated glass coverslips and grown to 95% confluence before being exposed to 0.1 mg/ml PM for 4 hrs. Control and/or PM-treated cells were fixed in formalin for 10 min before being permeabilized with 0.25% Triton X-100 for 5 minutes. ECs were blocked in 5% BSA followed by incubation with the Cx43 primary antibodies (Abcam) overnight at 4°C. The amount of Cx43-binding was detected by using Alexa 488-conjugated secondary antibodies (Life Technology). Cell nuclei were stained with DAPI (Thermo). The coverslips were mounted in Gold Antifade Mountant (Life Technology). Images were taken by using a Zeiss microscope, and excitation and emission wavelengths at 488 nm and 509 nm were used to capture the Cx43 signal, while excitation and emission wavelengths of 353 nm and 465 nm were used for DAPI. Cx43 subcellular localization was determined by confocal microscopy (Leica SP5), and the ER and Golgi apparatus were indicated by PDI antibodies (Abcam) and 58K (Abcam), respectively.

Microinjection of gap junction tracers

Gap junction activation was measured by tracing dye coupling. Endothelial cells were cultured on coverslips to 95% confluence, and then one single cell was implanted with a micropipette containing 5 mM (2-(4-nitro- 2,1,3-benzoxadiol-7-yl) [aminoethyl] trimethylammonium) (NBD-TMA) [21, 22]. The injection was conducted with the aid of a micromanipulator that allows for small movements [23, 24]. Images were collected using a fluorescence microscope in a time lapse for 5 min following injection. To determine channel activation, the number of dye-transferred cells was counted in a blinded manner and compared between PM-exposed and control samples by Student's t test.

Statistical analysis

Data are presented as the mean ± SEM for each experimental group. We performed statistical comparisons among treatment groups by randomized design and an unpaired Student's t-test for two groups. Statistical significance was defined as $P < 0.05$. These statistical analyses were performed by using GraphPad Prism software version 9.5.

Results

*PM induced Cx43 expression at the mRNA and protein levels*

In addition to tight junctions and adherens junctions, gap junctions belong to another type of intercellular junction that mediates adjacent cell communication by regulating small molecular-weight protein and ion passage. The gap junction channel is named a connexon, which is an apparatus assembled by six homo or hetero-connexin proteins. In endothelial cells, three kinds of connexins were observed: Cx43, Cx40 and Cx37 [25]. To investigate the Connexin expression pattern in human lung endothelial cells, we performed RT-PCR using gene-specific primers. Cx43 and Cx37 were abundantly expressed, but Cx40 was not observed (Figure S1A).

Our previous study demonstrated that particulate matter exposure disrupted the endothelial barrier and induced inflammatory lung injury [7-9, 26]. To determine whether Cx43 and Cx37 contribute to PM-induced barrier dysfunction, we investigated Cx43 and Cx37 mRNA expression patterns during the time course of challenge with 0.1 mg/ml PM. Real-time PCR showed that Cx43 constantly increased until 24 hrs after PM exposure, and there was a peak at 4 hrs showing more than 5 times that of the control (**Figure 1A**). Cx37 exhibited a fluctuating pattern, increasing to 2-fold at 1 hr, then dramatically reducing at 4 hr and maintaining the same level until 24 hr (Figure S1B). At the protein level, Cx37 was not altered by challenge with PM, while Cx43 expression was upregulated at the mRNA level (**Figure 1B**).
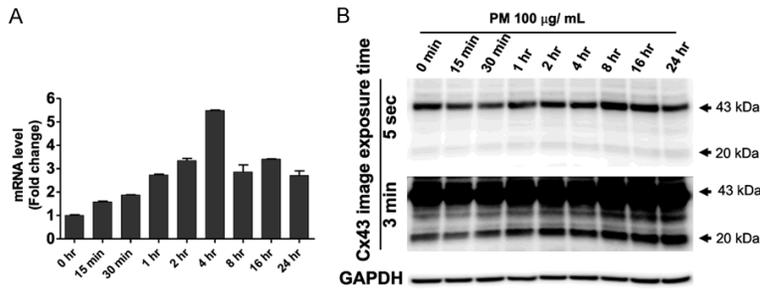
Figure 1. PM challenge mediates a continuously increased expression of Cx43 at both mRNA and protein levels. A. Real-time RT-PCR analysis confirms that Cx43 mRNA is increased by particulate matter (PM) (100 μg/ml) in a time-dependent manner. The bar graph indicates the relative mRNA level (vs. 0 hr) normalized to GAPDH. B. Western blot analysis of Cx43 proteins after PM stimulation (100 μg/mL) demonstrates time-dependent (0-24 hrs) increase of Cx43.
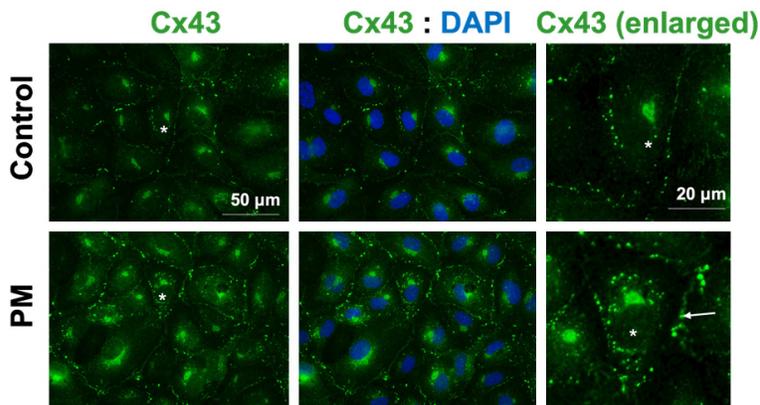
Figure 2. PM-induced Cx43 expression increase is determined by immunofluorescence staining. Distribution of Cx43 in control (upper) and 4 hrs PM-challenged (lower) ECs. Cx43 expression is detected by immunofluorescence (Green), and DAPI (Blue) is used to indicate cell nuclear. Images are a typical example of three separate experiments. The cell labeled with asterisk is shown enlarged on the right. Membrane associated and cytosolic Cx43 are both increased upon PM challenge. PM, Particulate Matter; ECs, endothelial cells.

was increased to 3 min. These results revealed that two Cx43 isoforms were present in human lung endothelial cells, and both proteins were upregulated by PM challenge.

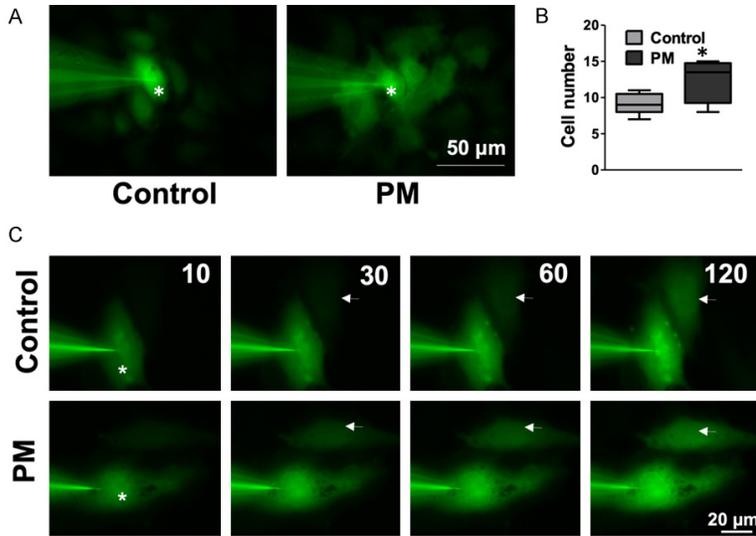*Cx43 was enhanced in both the cytosol and membranes*

Cx43 is a rapid turnover protein that is present in diverse subcellular locations. The Cx43 protein is synthesized in the endoplasmic reticulum and six individual proteins oligomerize to form hemichannels in the Golgi apparatus before assembling on the cell surface, in turn combining with adjacent cell hemichannels at intercellular plaques, which are termed full gap junction channels [11, 28]. To investigate the subcellular distribution of the PM-induced increase in Cx43, immunofluorescence analysis was conducted using a Cx43 C-terminal-specific antibody. In control monolayer cells, we observed gap junctional plaque-associated Cx43 at the membrane and intracellular Cx43 predominantly located at the perinucleus. After PM exposure for 4 hr, membrane and cytosolic proteins were dramatically enhanced (**Figure 2**), indicating that Cx43 protein expression was upregulated, and these data confirmed the western blot results. Taken together, PM upregulated cytosol and membrane Cx43 protein levels and increased cell-to-cell channel formation at adjacent cell plaques, suggesting an increase in channel activation.

PM increases gap junction channel activation

To determine whether the PM-induced increase in Cx43 expression alters intercellular communication, we microinjected single cells with NBD-TMA, a cationic dye used to test estimate

Multiple Cx43 isoforms have been identified in the human heart, and the 43 kDa and 20 kDa isoforms are predominant [27]. In the human lung endothelium, we observed two isoforms, a 43 kDa band named gap junction a1 - 43k (GJA1-43K) and a 20 kDa band named GJA1-20K, which could be clearly blocked by transfection with Cx43-specific siRNA (Figure S2). As **Figure 1B** shows, the 43 kDa band was much stronger than the 20 kDa band and was clearly increased after 8 hr of PM challenge with 5 sec of image exposure. In addition, the 20 kDa protein was also dramatically increased after 2 hr of PM challenge, and the image exposure time

Figure 3. PM-challenge enhances Gap junction communication competency compared to control ECs. Cell-to-cell communication was measured by microinjection of NBD-TMA in vehicle and PM-challenged for 4 hrs. A. Images are representative examples of NBD-TMA transfer in ECs. B. Bar graph indicates spread cell number after 5 min injection. PM challenge causes NBD-TMA transfer to more nearby cells. Error bars show SEM and *P < 0.05 versus vehicle. C. Time-lapse images sequence are recorded for 120 sec after injection. PM challenge causes more NBD-TMA transfer to nearby cells. Asterisks indicate the microinjected cells. White arrowhead indicates a nearby cell receiving transported NBD-TMA dye. PM, Particulate Matter; ECs, endothelial cells; NBD-TMA, (2-(4-nitro-2,1,3-benzoxadiol-7-yl) [aminoethyl] trimethylammonium).

Figure 4. N-acetyl-cysteine (NAC), a reactive oxygen species (ROS) scavenger, attenuates PM-increased Cx43 protein expression. Confluent ECs are pretreated with NAC (5 mM) for 1 hr and followed by PM exposure (100 µg/ml for 0-8 hr). Western blot data suggest that the increased pattern of Cx43 (both 43 kDa and 20 kDa) is blocked by NAC. ECs, Endothelial cells.
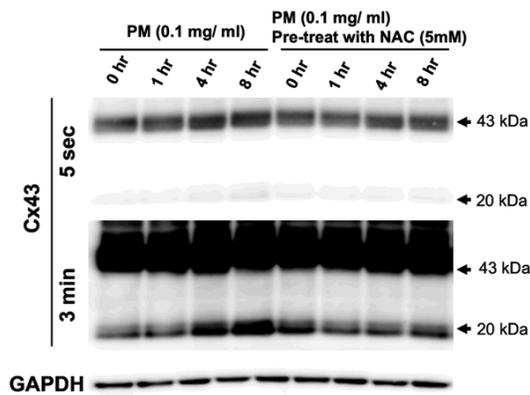
ed by capturing fluorescent digital images of cells every 5 sec during the first minute, followed by images at 2 min, 3 min, and 5 min. As shown in **Figure 3A**, during the 5 min, PM exposure for 4 hr led to a larger area of diffusion than control exposure. The number of recipient cells was counted to evaluate impacts on gap junction channels. Quantification analyses revealed that PM exposure increased the cell coupling number compared to that among control cells, and the extent of NBD-TMA was 12.5±3.1 in PM-treated cells and 9.2±1.4 in control cultures (**Figure 3B**).

The increase in fluorescence in recipient cells was termed the dye diffusion rate through gap junction channels. We observed that PM-treated cells showed a higher dye diffusion rate than control cells. The recipient cells showed visible fluorescence 30 sec after implantation, while control cells reached the same level at 60 sec (**Figure 3C**). These data indicated that PM exposure enhanced gap junction channel activation in endothelial cells.
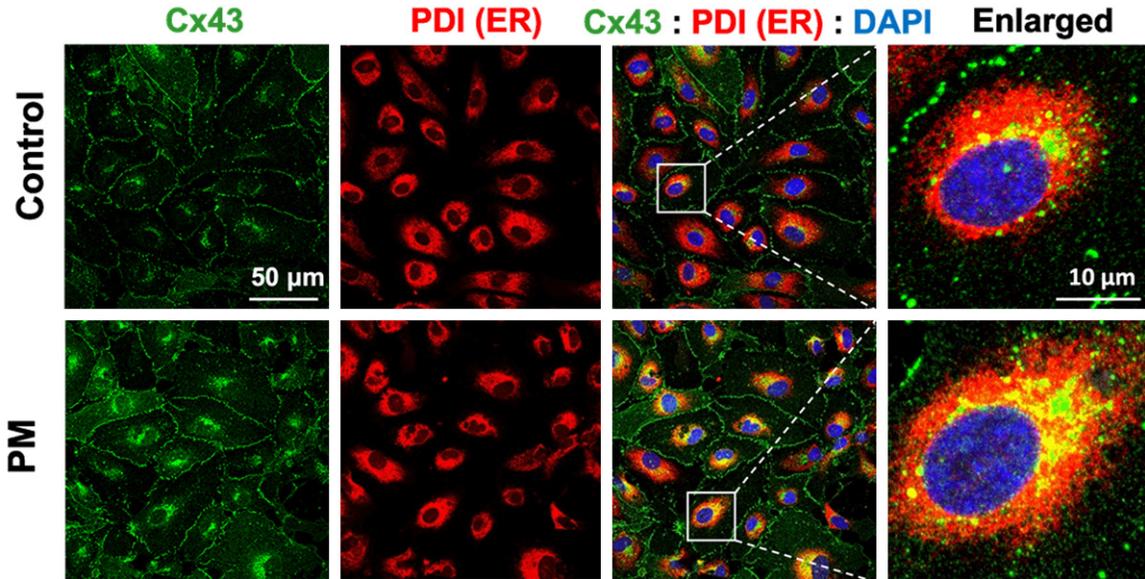
PM-induced Cx43 expression was increased through the ROS pathway

PM has been reported to stimulate oxidative stress in vascular endothelial cells by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [31], and we have demonstrated that PM-induced EC barrier disruption was mediated by an ROS-dependent pathway [7-9]. To determine whether the PM-induced increase in Cx43 expression was regulated by oxidative signaling, we used N-acetyl-cysteine (NAC), an ROS scavenger, to block ROS production. NAC pretreatment (5 mM, 1 hr) prevented the Cx43 (GJA1-43k and GJA1-20k) increase (both GJA1-43k and GJA1-20k) after 4 hr and 8 hr of PM exposure, and Cx43 was dramatically upregulated by PM treatment only (**Figure 4**). These results suggested that PM induced Cx43 transcription and protein production in an ROS-dependent manner.

gap junction permeability [22, 29] and charge-based selectivity of Cx43 [30]. The efficiency of dye transfer through adjacent cells was record-

Figure 5. PM-challenge induces Cx43 accumulation in endoplasmic reticulum (ER). Confluent ECs are treated with PM exposure (100 μg/ml for 4 hr) and fixed in 4% paraformaldehyde. Immunofluorescence is performed by using Cx43 antibody and ER specific marker PDI antibody, and compartment colocalization is imaged using Confocal (Leica SP5) at X 63 objective. PM, Particulate Matter.

PM challenge induced Cx43 accumulation in the ER/Golgi apparatus

It has been demonstrated that the truncated isoform GJA1-20k localizes to the perinuclear ER/Golgi apparatus and acts as a chaperone auxiliary protein to promote full-length GJA1-43K protein assembly in gap junction plaques, resulting in channel formation [27]. A detailed understanding of Cx43 cellular localization may uncover the mechanism of PM challenge in ECs. To identify Cx43 localization, high-resolution immunofluorescence analysis was performed using PDI and 58K proteins as biomarkers of the ER and Golgi apparatus, respectively. As **Figures 5** and **6** show, noncytosolic Cx43 protein colocalized with PDI and/or 58k protein. After PM stress for 4 hrs, more perinuclear Cx43 protein was produced and anchored in the ER and/or Golgi (**Figures 5** and **6**). PM regulates gap junction channel activation by stimulating GJA1-20K protein translation, which is then retained at the ER/Golgi, assisting GJA1-43K trafficking to cell plaques.

Cx43 contributed to PM-induced EC barrier disruption

Our previous experiment demonstrated that PM disrupts endothelial cell barrier integrity, resulting in enhanced organ dysfunction [7]. Cx43 participates in permeability alterations in ECs stressed by multiple inflammatory factors, such as thrombin and LPS [18]. We performed a TER assay to investigate whether Cx43 contributes to PM-induced endothelial permeability changes. Pretreatment with the selective Cx43 inhibitor GAP27 (60 nM) for 2 hr attenuated PM-induced barrier dysfunction by 45% (**Figure 7B**). This result was also verified by siRNA-mediated knockdown. The siRNA fragment targeting Cx43 was transfected into ECs, while the control had no siRNA, and there was significant ablation of the Cx43 protein (**Figure 7A**). Compared to control cells, Cx43 siRNA-treated cells showed 15% TER recovery under PM challenge, while Cx43 siRNA-treated cells without PM remained at baseline (**Figure 7A**).

To characterize the molecular mechanisms of Cx43-mediated EC barrier dysfunction, we examined the phosphorylation level of myosin light chain (Thr18 and Ser19), and the increase revealed paracellular gap formation that favored barrier disruption [32, 33]. In our study, PM challenge of human lung ECs for 15 min resulted in an increase in MLC phosphorylation at Thr18 and Ser19, and this phosphorylation remained at basal levels in PM-induced Cx43-knockout cells (**Figure 7B**). Taken together,
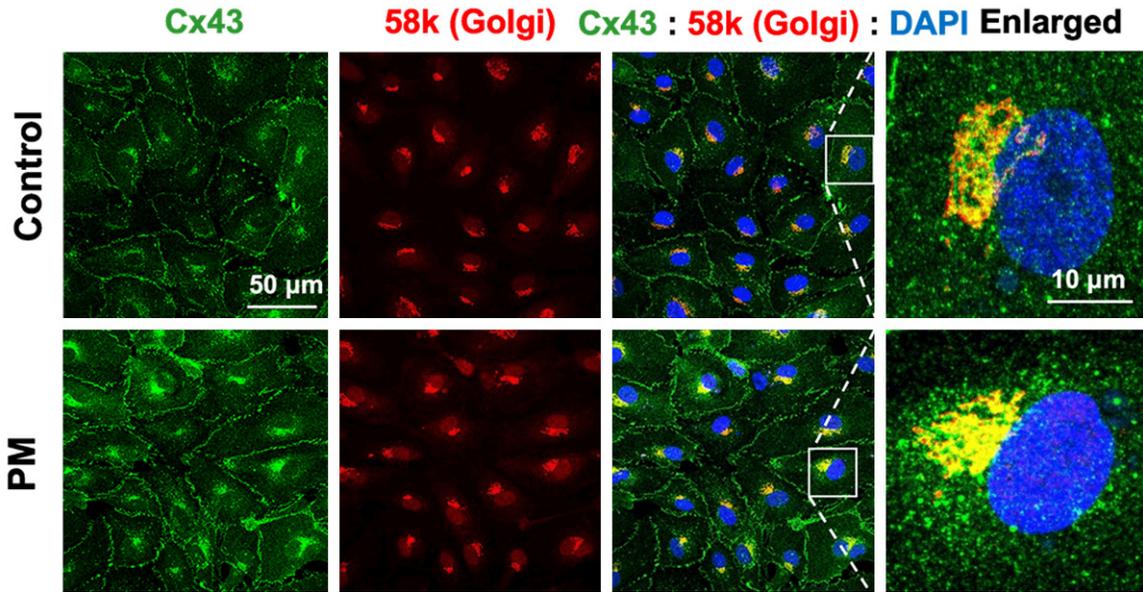
Figure 6. PM-challenge induces Cx43 accumulation in Golgi apparatus. Confluent ECs are treated with PM exposure (100 µg/ml for 4 hr) and fixed in 4% paraformaldehyde. Immunofluorescence is performed by using Cx43 antibody and Golgi specific marker 58K antibody, and compartment colocalization is imaged using Confocal (Leica SP5) at X 63 objective. These results indicate that PM-stress stimulates Cx43 vesicular transport and increase Gap Junction assembly. PM, Particulate Matter; ECs, endothelial cells.
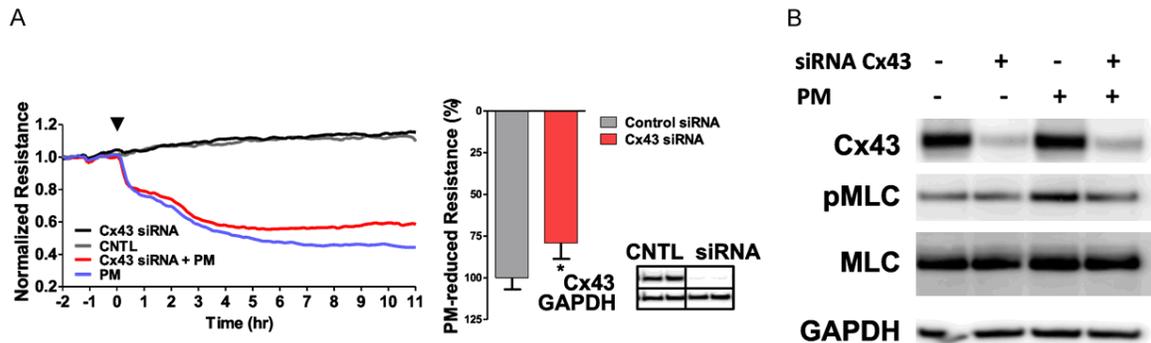
Figure 7. Blocking Cx43 activation abolishes PM-induced myosin light chain (MLC) phosphorylate, resulting in attenuation of PM-induced trans-endothelial electrical resistance (TER) reduction. A. ECs are transfected with specific siRNA of Cx43 leads to decline PM-induced ECs barrier disruption. TER is continually recorded for 11 hrs after PM exposure. Bar graphs indicate PM-induced TER reduction at 10 hr, and compared to non-inhibited ECs. Arrows indicate PM exposure (100 µg/ml). Western blot demonstrates Cx43 protein silencing. CNTL: Control. B. MLC phosphorylation level is stimulated by PM challenge (15 min) in ECs, while it is blocked in Cx43 silenced cells. PM, Particulate Matter; ECs, endothelial cells.

these results indicate that Cx43 contributes to PM-induced EC permeability by regulating MLC phosphorylation, which is a critical factor in cell contraction.

Discussion

Intercellular communication refers to the exchange of ions, metabolites, and water, which are essential for maintaining cellular function and signal transduction, and gap junction channels directly mediate this process. Gap junction channels form by connexin protein oligomerization, and six connexin proteins assemble into hexameric subunits termed connexons or hemichannels, which dock at cell plaques with complementary hemichannels provided by the apposed cell [34]. Connexin 43

(Cx43) is one of the most ubiquitously expressed gap junction proteins in vertebrate tissues [11]. In addition to mediating heart diseases, Cx43 has been characterized in various pulmonary inflammatory diseases, such as acute lung injury, cystic fibrosis and asthma [13, 35, 36]. Vascular endothelial cells act as a semiselective barrier to resist devastating inflammatory syndromes by preventing fluid and solute flux across the blood vessel wall. Increased vascular permeability resulting from the disruption of EC barrier integrity is a cardinal feature of inflammatory lung injury [37].

In the present study, we found that Cx43 was expressed in human lung endothelial cells and was upregulated by PM stress in an ROS-dependent manner. Increased Cx43 aggregated in the cell membrane through the ER/Golgi vesicular transport pathway, increasing gap junction channel formation, which in turn enhanced channel activation. We also demonstrated that Cx43 contributes to PM-induced EC barrier disruption by controlling MLC phosphorylation. Our results indicated that Cx43 mediates the PM-induced EC permeability increase.

Cx43 contains four transmembrane domains (M1, M2, M3 and M4), two extracellular loops (E1 and E2), one intracellular loop (IL) and the amino and carboxy-terminal parts (NT and CT, respectively), which are intracellular [38]. A recent study described multiple Cx43 isoforms generated by the same mRNA sequence in human heart tissue, and 43 kDa and 20 kDa were the predominant forms. The 20 kDa isoforms docked at the ER/Golgi apparatus and was essential for full-length protein trafficking to the cell plaque to build a complete gap junction channel [27]. Similar to that in heart tissue, we observed two Cx43 isoforms in human lung ECs (43 kDa and 20 kDa) by immunoblot analysis, and both of these proteins were upregulated by PM stimulation over time. These results were also confirmed by high-resolution immunofluorescence images. After PM stress for 4 hrs, in addition to an increase in cell membrane proteins, an enhanced signal was observed at the ER and/or Golgi apparatus, suggesting that PM upregulated the two isoforms of Cx43, while the 20 kDa isoform served as a chaperone protein that assisted transport of the 43 kDa protein (may appear as a hexamer subunit) to inter-

calated discs and to form a fully activated channel. Cx43 can be directly delivered to the plasma membrane by dynamic cytoskeletal proteins, including the microtubule plus-end tracking proteins EB1 and p150 (Glued) and β-actin [39-41]. It would be intriguing to verify the mechanism by which the 20 kDa isoform protein facilitates Cx43 trafficking to cell-cell junctions in EC.

We and others have found that PM increases intracellular and/or extracellular ROS generation, resulting in the direct induction of oxidative stress in vascular endothelial cells [7, 31, 42]. Here, we reported that the PM-induced increase in Cx43 was an underlying ROS-dependent process in EC. In cultured cardiomyocytes, exposure to the ROS hydrogen peroxide (H_2O_2) induced a ROS-Cx43 positive feedback loop in which ROS upregulated Cx43 expression and Cx43 induced intracellular ROS production [43]. However, whether Cx43 acts as a protective or detrimental factor in response to ROS-induced oxidative stress is still under debate [44, 45]. In our previous study, PM-induced ROS production was shown to be directly involved in EC barrier dysfunction [7]; currently, we demonstrated that inhibition of Cx43 attenuated the PM-induced decrease in EC barrier function. We anticipate that a similar positive feedback loop may occur in ECs in response to PM challenge. The increase in Cx43, which is regulated by PM exposure in an ROS-dependent manner, supported ROS accumulation.

The key event in endothelial barrier disruption properties the increase in MLC phosphorylation leading to cell contraction [32]. Cx43 exacerbates human lung vascular permeability induced by thrombin, a barrier-disrupting protease [18, 46], and this effect may be modulated by increased MLC phosphorylation, which alleviates Cx43 blockade activation [18]. Similarly, in rat pulmonary vein vascular endothelial cells, Cx43 increases vascular permeability by increasing MLC phosphorylation by the activity of the RhoA/RACK kinase pathway [17]. In our study, Cx43 knockdown attenuated PM-induced MLC phosphorylation; however, further investigation is needed to determine whether this effect is mediated by RhoA kinase or Ca^{2+}/calmodulin-dependent myosin light chain kinase signaling. PM induces calcium
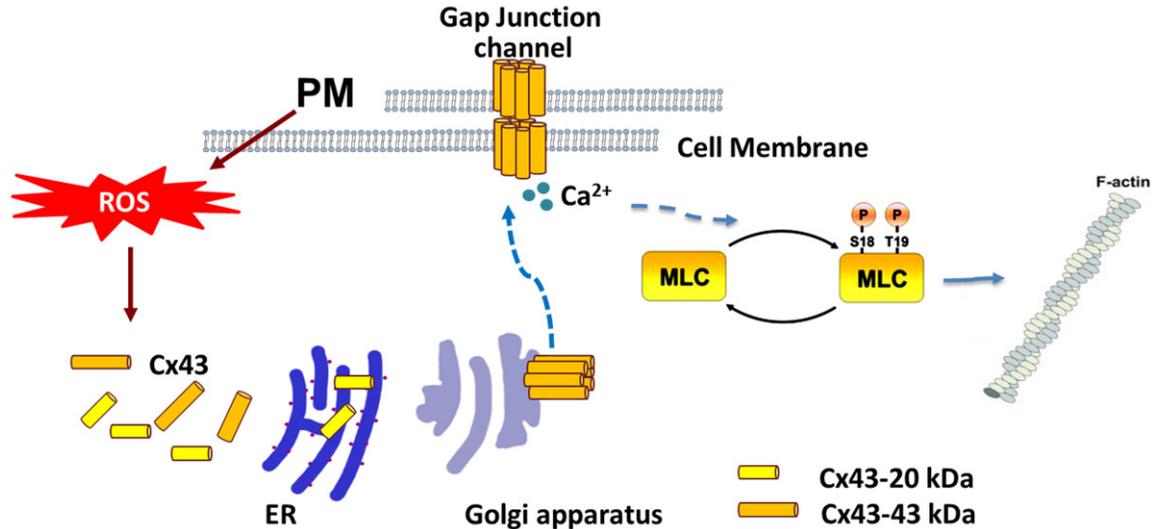
Figure 8. Hypothesized mechanism of the effects of PM challenge on EC Cx43. PM stimulates ROS generation in ECs, in turn leading to Cx43 transcription activation and protein expression increase. PM-challenge induces Cx43 vesicular transport by ER and Golgi pathway and more Cx43 protein locates to cell plaque for assessment more connexon channels which implement intercellular communication function, including Calcium (Ca^{2+}) flux. Calcium regulates MLC phosphorylate level that mediates the cell barrier directly.

leakage in EC [9], and Cx43 is a critical factor that induces secondary Ca^{2+} increases in the adjacent capillary networks in mice [46]. We demonstrated that cellular communication was enhanced by PM exposure. Therefore, we hypothesize that Cx43 may spread the PM-induced proinflammatory response by controlling Ca^{2+} release.

Based on previously reported data and our present results, we suggest a detrimental role of Cx43 in PM-induced EC permeability (**Figure 8**). PM exposure upregulates the expression of the gap junction protein Cx43, both the full-length 43 kDa isoform and the truncated 20 kD isoform. The ER/Golgi apparatus-associated 20 kDa protein serves as a chaperone to facilitate hexamer polymerization of the 43 kDa isoform and transport to intercalated discs to form an integrated gap junction channel with another hemichannel provided by the opposed cell. Increased channel activation enhances PM-induced inflammatory spread by stimulating Ca^{2+} in adjacent cells, resulting in MLC phosphorylation and modulating cytoskeletal arrangement and cell contraction. These processes are under the oxidative-dependent signaling pathway. The ability of PM to regulate Cx43 function provides a mechanistic framework for understanding how Cx43 participates in lung injury and diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liming Zhang, Department of Respiratory and Critical Care Medicine, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China. Tel: +86-10-51718813; E-mail: cyyyzlm@sina.com; Dr. Ting Wang, Center of Translational Science, Florida International University, 11350 SW Village Parkway, Port St. Lucie, FL 34987, USA. Tel: 772-345-4716; E-mail: tinwang@fiu.edu

References

[1] Ristovski ZD, Miljevic B, Surawski NC, Morawska L, Fong KM, Goh F and Yang IA. Respiratory health effects of diesel particulate matter. Respirology 2012; 17: 201-212.

[2] Anderson JO, Thundiyil JG and Stolbach A. Clearing the air: a review of the effects of particulate matter air pollution on human health. J Med Toxicol 2012; 8: 166-175.

[3] Brook RD, Rajagopalan S, Pope CA 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC Jr, Whitsel L and Kaufman JD; American Heart Association Council on Epidemiology and Prevention, Council on the Kidney in Cardiovascular Disease, and Council on Nutrition, Physical Activity and Metabolism. Par-

ticulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. Circulation 2010; 121: 2331-2378.

[4] Pope CA 3rd and Dockery DW. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 2006; 56: 709-742.

[5] Nel A, Xia T, Madler L and Li N. Toxic potential of materials at the nanolevel. Science 2006; 311: 622-627.

[6] Wang T, Moreno-Vinasco L, Huang Y, Lang GD, Linares JD, Goonewardena SN, Grabavoy A, Samet JM, Geyh AS, Breysse PN, Lussier YA, Natarajan V and Garcia JG. Murine lung responses to ambient particulate matter: genomic analysis and influence on airway hyperresponsiveness. Environ Health Perspect 2008; 116: 1500-1508.

[7] Wang T, Chiang ET, Moreno-Vinasco L, Lang GD, Pendyala S, Samet JM, Geyh AS, Breysse PN, Chillrud SN, Natarajan V and Garcia JG. Particulate matter disrupts human lung endothelial barrier integrity via ROS- and p38 MAPK-dependent pathways. Am J Respir Cell Mol Biol 2010; 42: 442-449.

[8] Wang T, Wang L, Zaidi SR, Sammani S, Siegler J, Moreno-Vinasco L, Mathew B, Natarajan V and Garcia JG. Hydrogen sulfide attenuates particulate matter-induced human lung endothelial barrier disruption via combined reactive oxygen species scavenging and Akt activation. Am J Respir Cell Mol Biol 2012; 47: 491-496.

[9] Wang T, Wang L, Moreno-Vinasco L, Lang GD, Siegler JH, Mathew B, Usatyuk PV, Samet JM, Geyh AS, Breysse PN, Natarajan V and Garcia JG. Particulate matter air pollution disrupts endothelial cell barrier via calpain-mediated tight junction protein degradation. Part Fibre Toxicol 2012; 9: 35.

[10] Wei CJ, Xu X and Lo CW. Connexins and cell signaling in development and disease. Annu Rev Cell Dev Biol 2004; 20: 811-838.

[11] Falk MM, Bell CL, Kells Andrews RM and Murray SA. Molecular mechanisms regulating formation, trafficking and processing of annular gap junctions. BMC Cell Biol 2016; 17 Suppl 1: 22.

[12] Figueroa XF and Duling BR. Gap junctions in the control of vascular function. Antioxid Redox Signal 2009; 11: 251-266.

[13] Freund-Michel V, Muller B, Marthan R, Savineau JP and Guibert C. Expression and role of connexin-based gap junctions in pulmonary inflammatory diseases. Pharmacol Ther 2016; 164: 105-119.

[14] Johnson LN and Koval M. Cross-talk between pulmonary injury, oxidant stress, and gap junc-

tional communication. Antioxid Redox Signal 2009; 11: 355-367.

[15] Kim J, Hwangbo C, Hu X, Kang Y, Papangeli I, Mehrotra D, Park H, Ju H, McLean DL, Comhair SA, Erzurum SC and Chun HJ. Restoration of impaired endothelial myocyte enhancer factor 2 function rescues pulmonary arterial hypertension. Circulation 2015; 131: 190-199.

[16] Zhang J, Wang W, Sun J, Li Q, Liu J, Zhu H, Chen T, Wang H, Yu S, Sun G, Chen W and Yi D. Gap junction channel modulates pulmonary vascular permeability through calcium in acute lung injury: an experimental study. Respiration 2010; 80: 236-245.

[17] Zhang J, Yang GM, Zhu Y, Peng XY, Li T and Liu LM. Role of connexin 43 in vascular hyperpermeability and relationship to Rock1-MLC20 pathway in septic rats. Am J Physiol Lung Cell Mol Physiol 2015; 309: L1323-1332.

[18] O'Donnell JJ 3rd, Birukova AA, Beyer EC and Birukov KG. Gap junction protein connexin43 exacerbates lung vascular permeability. PLoS One 2014; 9: e100931.

[19] Walters DM, Breysse PN and Wills-Karp M. Ambient urban Baltimore particulate-induced airway hyperresponsiveness and inflammation in mice. Am J Respir Crit Care Med 2001; 164: 1438-1443.

[20] Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR and English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. J Clin Invest 2001; 108: 689-701.

[21] Bednarczyk D, Mash EA, Aavula BR and Wright SH. NBD-TMA: a novel fluorescent substrate of the peritubular organic cation transporter of renal proximal tubules. Pflugers Arch 2000; 440: 184-192.

[22] Ek-Vitorin JF and Burt JM. Quantification of gap junction selectivity. Am J Physiol Cell Physiol 2005; 289: C1535-1546.

[23] Lampe PD, Cooper CD, King TJ and Burt JM. Analysis of Connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart. J Cell Sci 2006; 119: 3435-3442.

[24] Gemel J, Nelson TK, Burt JM and Beyer EC. Inducible coexpression of connexin37 or connexin40 with connexin43 selectively affects intercellular molecular transfer. J Membr Biol 2012; 245: 231-241.

[25] Bazzoni G and Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev 2004; 84: 869-901.

[26] Wang T, Lang GD, Moreno-Vinasco L, Huang Y, Goonewardena SN, Peng YJ, Svensson EC, Natarajan V, Lang RM, Linares JD, Breysse PN, Geyh AS, Samet JM, Lussier YA, Dudley S, Prab-

hakar NR and Garcia JG. Particulate matter induces cardiac arrhythmias via dysregulation of carotid body sensitivity and cardiac sodium channels. Am J Respir Cell Mol Biol 2012; 46: 524-531.

[27] Smyth JW and Shaw RM. Autoregulation of connexin43 gap junction formation by internally translated isoforms. Cell Rep 2013; 5: 611-618.

[28] Koval M. Pathways and control of connexin oligomerization. Trends Cell Biol 2006; 16: 159-166.

[29] Ek-Vitorin JF, King TJ, Heyman NS, Lampe PD and Burt JM. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ Res 2006; 98: 1498-1505.

[30] Heyman NS and Burt JM. Hindered diffusion through an aqueous pore describes invariant dye selectivity of Cx43 junctions. Biophys J 2008; 94: 840-854.

[31] Li Z, Hyseni X, Carter JD, Soukup JM, Dailey LA and Huang YC. Pollutant particles enhanced H2O2 production from NAD(P)H oxidase and mitochondria in human pulmonary artery endothelial cells. Am J Physiol Cell Physiol 2006; 291: C357-365.

[32] Dudek SM and Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol (1985) 2001; 91: 1487-1500.

[33] Wang P, Verin AD, Birukova A, Gilbert-McClain LI, Jacobs K and Garcia JG. Mechanisms of sodium fluoride-induced endothelial cell barrier dysfunction: role of MLC phosphorylation. Am J Physiol Lung Cell Mol Physiol 2001; 281: L1472-1483.

[34] Nielsen MS, Axelsen LN, Sorgen PL, Verma V, Delmar M and Holstein-Rathlou NH. Gap junctions. Compr Physiol 2012; 2: 1981-2035.

[35] Yao Y, Zeng QX, Deng XQ, Tang GN, Guo JB, Sun YQ, Ru K, Rizzo AN, Shi JB and Fu QL. Connexin 43 upregulation in mouse lungs during ovalbumin-induced asthma. PLoS One 2015; 10: e0144106.

[36] Islam MN, Das SR, Emin MT, Wei M, Sun L, Westphalen K, Rowlands DJ, Quadri SK, Bhattacharya S and Bhattacharya J. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nat Med 2012; 18: 759-765.

[37] Chiang ET, Wang T and Garcia JGN. Acute lung injury: the injured lung endothelium, therapeutic strategies for barrier protection, and vascular biomarkers. Textbook of Pulmonary Vascular Disease 2011; 197-222.

[38] Yeager M and Harris AL. Gap junction channel structure in the early 21st century: facts and fantasies. Curr Opin Cell Biol 2007; 19: 521-528.

[39] Smyth JW, Hong TT, Gao D, Vogan JM, Jensen BC, Fong TS, Simpson PC, Stainier DY, Chi NC and Shaw RM. Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. J Clin Invest 2010; 120: 266-279.

[40] Shaw RM, Fay AJ, Puthenveedu MA, von Zastrow M, Jan YN and Jan LY. Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. Cell 2007; 128: 547-560.

[41] Smyth JW, Vogan JM, Buch PJ, Zhang SS, Fong TS, Hong TT and Shaw RM. Actin cytoskeleton rest stops regulate anterograde traffic of connexin 43 vesicles to the plasma membrane. Circ Res 2012; 110: 978-89.

[42] Montiel-Davalos A, Ibarra-Sanchez Mde J, Ventura-Gallegos JL, Alfaro-Moreno E and Lopez-Marure R. Oxidative stress and apoptosis are induced in human endothelial cells exposed to urban particulate matter. Toxicol In Vitro 2010; 24: 135-141.

[43] Kawahara K and Matsuyama D. Positive-feedback regulation loop for the loss of cell division and binucleation shortly after birth in cardiomyocytes. 5th European Conference of the International Federation for Medical and Biological Engineering, Pts 1 and 2 2012; 37: 423-+.

[44] Le HT, Sin WC, Lozinsky S, Bechberger J, Vega JL, Guo XQ, Saez JC and Naus CC. Gap junction intercellular communication mediated by connexin43 in astrocytes is essential for their resistance to oxidative stress. J Biol Chem 2014; 289: 1345-1354.

[45] Ramachandran S, Xie LH, John SA, Subramaniam S and Lal R. A novel role for connexin hemichannel in oxidative stress and smoking-induced cell injury. PLoS One 2007; 2: e712.

[46] Parthasarathi K, Ichimura H, Monma E, Lindert J, Quadri S, Issekutz A and Bhattacharya J. Connexin 43 mediates spread of Ca2+-dependent proinflammatory responses in lung capillaries. J Clin Invest 2006; 116: 2193-2200.
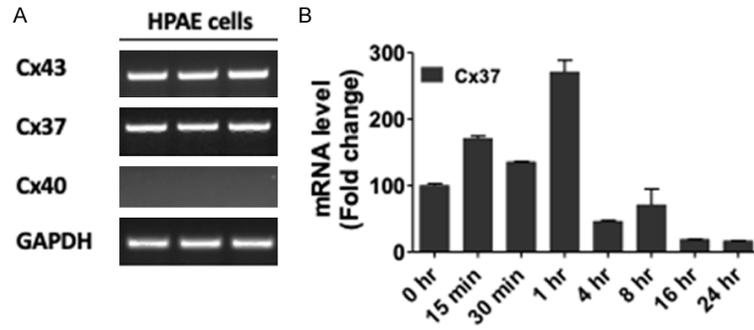
Figure S1. A. RT-PCR confirms that Cx37 and Cx43 are abundantly expressed in human pulmonary endothelial cells (HPAECs), but Cx40 was not detected. B. Cx37 mRNA expression fluctuates under PM stress.
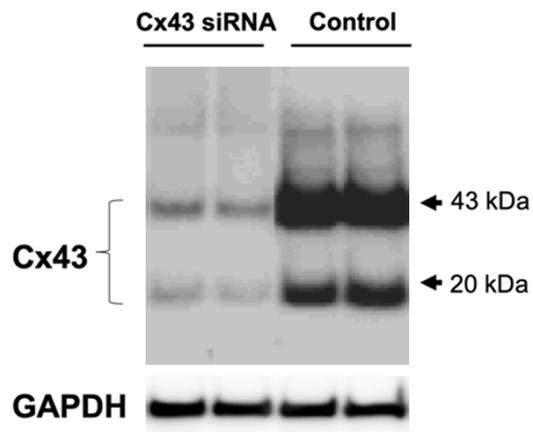
Figure S2. Cx43-specific siRNA abolishes Cx43 kDa and 20 kDa isoforms.